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Phytochemistry 63 (2003) 249–255
PHYTOCHEMISTRY
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Cys/Gly-rich proteins with a putative single chitin-binding domain from oat (*Avena sativa*) seeds

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Received 26 July 2002; received in revised form 3 February 2003

Abstract

Through a reliable and repeatable procedure based on solid-phase extraction techniques, a protein fraction (P fraction) rich in Cys/Gly residues was extracted and captured from oat (*Avena sativa* L.) seeds. Quantitative amino acid analysis and MS of the P fraction indicated that it contains a series of heterogeneous Cys/Gly-rich proteins with molecular masses of 3.6–4.0 kDa. Preliminary results from bioassays showed that these proteins possess weak to moderate antifungal properties to some fungal strains. From this fraction, a new polypeptide, designated avesin A, was purified and sequenced by Edman degradation. Avesin A consists of 37 amino-acid residues, with 10 glycine residues and eight cysteine residues forming disulfide bridges, and contains a single chitin-binding domain, which indicates that avesin A is a new member of the putative chitin-binding proteins. Avesin A is the first identified hevein-like small protein from cereal grains.

Keywords: *Avena sativa*; Gramineae; Oat; Isolation and identification; Polypeptide; Avesin A

1. Introduction

To protect themselves from pathogen invasion, plants produce a wide array of antimicrobial compounds to reduce the potential for invasion. Proteins with antifungal activities form a very important part of these defenses. Traditionally, these proteins include ribosome-inactivating proteins (RIPs) (Peumans et al., 2001), hydroxyproline-rich glycoproteins, proteinase inhibitors, enzymes for the synthesis of phytoalexins, enzymes contributing to the reinforcement of cell walls, and certain hydrolytic enzymes such as glucanases and chitinases (Zhu and Lam, 1997). As demonstrated in recent years, a variety of small proteins/polypeptides (20–60 amino acid residues) are involved in plant inducible defenses by functioning as intra-/inter-cellular signaling molecules or as extracellular defenders (Bergey et al., 1996; Broekaert et al., 1997; Franssen and Bisseling, 2001; Ryan and Pearce, 2001). These Cys-rich polypeptides, though small, are highly divergent in primary and secondary structure, and exhibit quite different antibiotic activities (Broekaert et al., 1997). Among them, plant defensins and thionins, found ubiquitously in cereal crops and believed to play a defense role in restricting pathogen growth (Broekaert et al., 1997; Garcia-Olmedo et al., 2001), have been collectively referred to as PR-12 and PR-13, members of the pathogenesis-related proteins (PRs) (Van Loon and Van Strien, 1999). We have now discovered another class of Cys/Gly-rich PR-like proteins (PRLs), hevein-like chitin-binding small proteins that exist in cereal grains, at least in oat seeds.

In this report we describe a simple, reliable procedure based on solid-phase extraction techniques for the fractionation of these small proteins from oat seeds. We also report the isolation (from the fraction) of the first

Abbreviations: AAA, amino acid analysis; RP, reverse-phase; HPLC, high-performance liquid chromatography; t_R, retention time; DAD, diode array detector; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; TOF, time-of-flight; TFA, trifluoroacetic acid; PRS, propylsulphonic acid; DTE, dithioerythritol; EDTA, ethylenedinitrilotetracetic acid; PEC, *S*-(β-4-pyridylethyl) cysteine.

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0031-9422/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00116-X

hevein-like small protein, designated avesin A, and describe its structural determination.

2. Results

2.1. Purification of new polypeptides

Based on solid-phase extraction procedures, a fraction enriched in polypeptides (P fraction) obtained from the oat seeds, appeared as a white powder. Quantitative AAA revealed that this fraction mainly contained proteins or polypeptides with extremely high ratios of glycine and cysteine residues. The P fraction was purified further by cation exchange chromatography, and the resulting profile showed that it comprised several constituents (Fig. 1). Quantitative AAA of the three collected fractions indicated that most polypeptides were eluted after t_R 25 min, while the peaks before t_R 20 min were predominantly non-polypeptide components. The polypeptides in the fraction with t_R 25–30 min were pooled together and subjected to repeated RP-HPLC resulting finally in the isolation of a homogeneous polypeptide. The inset in Fig. 1 shows the polypeptide's RP-HPLC profile (retention time) from the final purification step.

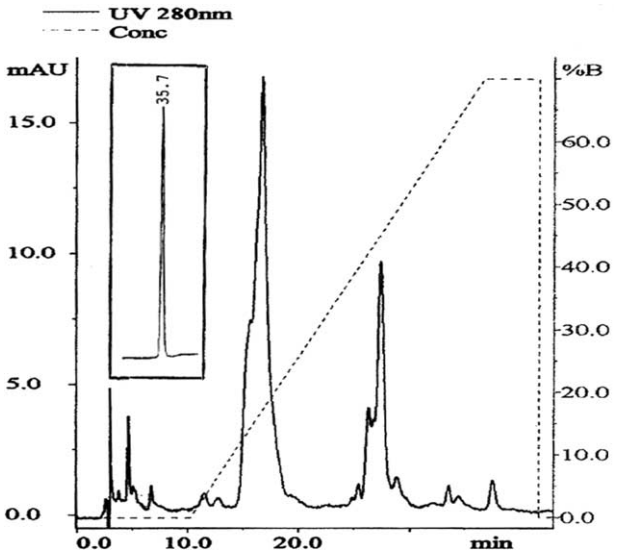

Fig. 1. Chromatography profiles of the P fraction and avesin A. The purification of avesin A was achieved by cation-exchange liquid chromatography followed by RP-HPLC. In cation-exchange chromatography, two 50/7.5 (i.d.) mm Vydac Protein SCX columns were directly connected and eluted at a flow rate of 1.0 ml/min with a programmed gradient from 100% eluent A to 70% eluent B (cf. Experimental). A: 15% CH_3CN, 0.1% TFA in water; B: 15% CH_3CN, 0.1% TFA, 0.1 M NaCl in water. The major profile shows the cation-exchange chromatogram. The fraction collected between $t_R = 25$ min and $t_R = 30$ min was repeatedly purified by RP-HPLC. In the final purification, a 250/4.6 (i.d.) mm Rainin Dynamax column (C_{18}, 5 μm, 300 Å) was used and eluted with a linear gradient of 5–40% organic modifier (CH_3CN/*i*-PrOH, 6/4) in aqueous 0.1% TFA during 50 min at a flow rate of 0.8 ml/min. Avesin A ($t_R = 35.7$ min) was thus obtained and its UV spectrum recorded by DAD. The HPLC profile of avesin A from the final purification step is shown as an inset.

2.2. Mass spectrometric analysis and amino acid sequence

MALDI-MS data showed that the P fraction from the oat seeds consisted of a series of heterogeneous components with molecular weights from 3.6 to 4.0 kDa. After ion exchange and repeated C_{18} HPLC, a homogeneous polypeptide resulted from the P fraction. MALDI- and ESI-MS analysis of the polypeptide gave an average mass of 3684 Da and a monoisotopic mass of 3681 Da, respectively. By quantitative AAA, a high ratio of cysteine residues (eight residues per mole) was found in the isolated polypeptide; and therefore a portion of the re-dissolved protein was reduced and alkylated for direct Edman sequencing. The experimentally (MALDI-TOF) determined average mass for the PEC derivative was 4533 and 4428 Da. The mass of 4533 Da accords well with the presumption that all the eight cysteines engaged in disulfide bridges in the native protein are reduced and alkylated in the derivative. The mass of 4428 Da is consistent with the theoretically calculated mass of the derivative, with seven alkylated cysteine residues, suggesting that partial alkylation co-existed in the reaction.

The PEC derivatives were then sequenced using the method of automated Edman degradation. The first three cycles gave a clear and single amino acid, read manually, as 1—WSG—3. The fourth cycle resulted in a blank as the first choice, and a Ser residue (about 10% of the signal in the third cycle) as the second-choice amino acid. The other 33 cycles gave the following sequence: 5-SPCPGNECCSKYGYCGLGGDYCGAGCQSGPCYG-37. A second-choice signal (about 10% of the main signal) was also read between cycle 5 and cycle 16, giving exactly the same sequence as that of the main signal from cycle 6 to cycle 17. Based on the data from quantitative AAA and MS, together with the fact that partial alkylation existed in the PEC derivatives, we concluded that the fourth amino acid in the polypeptide chain was a Cys residue. We concluded that the observed second-choice low signal from cycle 4 to cycle 16 was a degradation product of the main sequence: the cleavage site in the native protein was inferred from this signal to be between residues 5 and 6. The reduced and non-alkylated Cys in the fourth cycle may have facilitated the cleavage, which occurred during the fourth cycle. The amino acid sequence of the polypeptide (1-WSGCSPCPGNECCSKYGYCGLGGDYCGAGCQSGPCYG-37) was thus determined. The identified polypeptide, named avesin A (after its origin), is a new small protein. Avesin A consists of 37 amino acids, with two negatively charged residues (1E and 1D) and one positively charged residue (1K), giving a theoretical pI of 4.4 (calculated through the program at:

http://www.expasy.ch/tools/pi_tool.html) (Bjellqvist et al., 1993, 1994). Avesin A is rich in Gly (10G) and Cys (8C) residues, which is another striking feature. The mass, calculated from the primary sequence, was 8 Da higher than the apparent molecular weight, again in agreement with the arrangement of eight Cys residues to form intramolecular disulfide bridges within the native protein, a process that eliminates eight hydrogen atoms.

2.3. Database search and sequence alignment

The sequence of avesin A was used as an initial query for the iterated bioinformatics search against the non-redundant (NR) database. More than 250 sequences (DNA-derived or biochemical identified), retrieved with statistically significant *E* values, were extracted from eukaryotic proteins (the data mining was conducted on 28 November 2001; data not shown). Taxonomically, those sequences are widely distributed, having been found in seed plants (as the majority), including eudicots and monocots; in the fungal species, *Aspergillus nidulans*, *Blumeria graminis*, *Kluyveromyces lactis*, and *Pichia etchellsii*; and in the nematode, *Caenorhabditis elegans*. Among the flowering plants, many taxa are of economic and agronomic importance, such as the potato, tomato, tobacco, bean, pea, carrot, chestnut, rape, cane, banana, garlic, yam, and most grain species.

By further analyzing the sequence of avesin A against the Pfam and SMART databases, we classified avesin A as a new member of the chitin recognition proteins (Pfam accession number: PF00187), which may contain a single chitin-binding domain, the ChtBD1 (SMART accession number: SM0270), or multiple domains. The ChtBD1 domain-containing proteins of plant origin consist of chitinases/endochitinases, chitin-binding lectins, and hevein-like small proteins. The domain is 29–45 amino acids in length, and is either repeated twice (e.g., nettle lectin), 4-fold (e.g., wheat, barley and rice lectins), or fused to an unrelated domain (e.g., basic chitinases and prohevein), while the hevein-like small proteins essentially comprise only the conserved domain. Thus, the sequence of avesin A, aligned with the selected items, resulted from the PSI-BLAST search as shown in Fig. 2.

The result of the alignment indicates that the ChtBD1 plant domains are rich in Cys/Gly residues, and that the amino acid residues in the central region of the domain are well conserved. The highly conserved Cys residues suggest that they may have similar 3D structures. In contrast, most gaps are present in the N- and C-terminal regions, which are less conserved. Avesin A shares with chitin-binding plant proteins the conserved motif—the triad of aromatic residues, a Ser, and a Glu/Asp—involved in saccharide (chitin/*N*-acetylglucosamine) binding (Raikhel et al., 1993), implying that the avesins may possess antifungal and/or insecticidal properties. Experimentally, the P fraction showed weak inhibition of spore germination of the fungus *Drechslera sorokiniana* at the concentration of 500 μg/ml using the bioassays described by Thaning et al. (2001). The desalted polypeptide fractions (mainly containing avesin A) isolated by cation exchange chromatography (t_R, 25-30 min) moderately inhibited spore germination of the fungus *D. sorokiniana* (concentration, 200 μg/ml), in a dose-dependent manner, and weakly inhibited spore germination of the fungus *Fusarium culmorum* (concentration, 300 μg/ml) (J.J. Borowicz, C.J. Welch, S.-S. Li, P. Claeson, unpublished observation), which merits further investigation of possible antifungal properties of this group of small proteins from oat.

3. Discussion

The procedures for fractionation and purification of small proteins from oat seeds described in this paper (which we have repeatedly practiced) appear reliable and reproducible. Gly-rich small proteins are generally very hydrophilic; and thus our protocol, based on solid-phase extraction and RP-HPLC chromatography methods, can be further applied to the detection and isolation of Gly/Cys-rich proteins, such as small chitin-binding proteins from other plant biomass. The isolated and sequenced avesin A represents the first hevein-like small protein from cereal crops. Other hevein homologues, such as Ac-AMPs from *Amaranthus caudatus* (Broekaert et al., 1996), Pn-AMPs from *Pharbitis nil* (Koo et al., 1998), and GAFP from *Ginkgo biloba* (Huang et al., 2001), have been reported in the last decade, after hevein was first discovered from the latex of the rubber tree by Archer (1960). Although the chitinases/endochitinases, chitin-binding lectins, and hevein-like proteins share the same hevein domain (ChtBD1), they do not adopt the same antimicrobial mode of action, due to differences in their subcellular locations, molecular sizes, and overall structures (Beintema, 1994; Raikhel et al., 1993).

Whether hevein and avesin A share the same antifungal mode of action with their basic homologues is unclear. The 3D structure of hevein shows that most of its basic amino acid residues are located around the chitin-binding motif, with the orientation opposing the clustered acidic residues (Andersen et al., 1993). Avesin A has a modeled 3D structure similar to that of hevein—an intriguing feature—disclosed by homology modeling using the SWISS-MODEL program (Baker and Sali, 2001; Guex et al., 1999).

Among the hevein homologues, Ac-AMPs, Pn-AMPs, and GAFP, which are all highly basic and have pI values above 10, have shown a wide range of in vitro antifungal-activity potencies. Their activities, which were found to be strongly dependent on the ionic composition of the

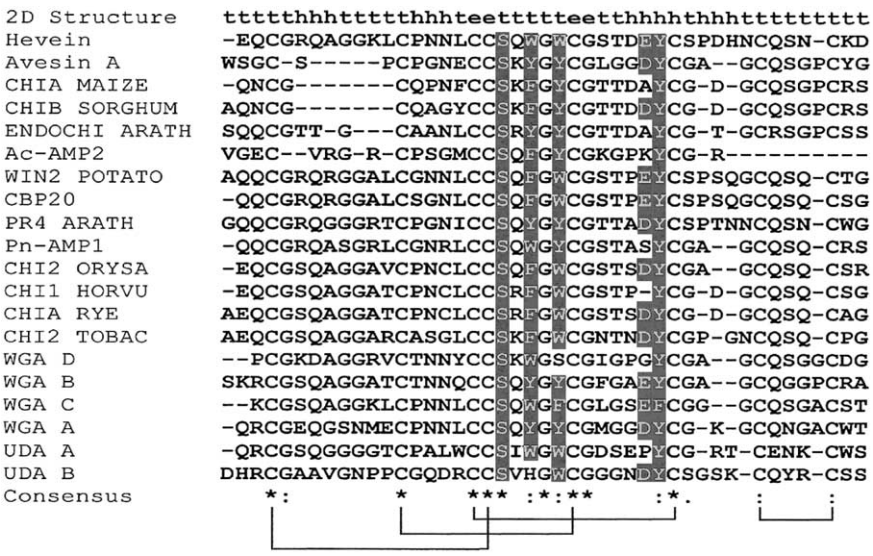

Fig. 2. Alignment of selected amino-acid sequences of the chitin-binding domains. The one-letter code for amino acids is used in the sequence lines. The sequences were aligned using the CLUSTAL W (1.81) program, and the alignment was refined manually to maximize sequence conservation for each individual sequence. Gaps are represented by dashes. Amino acid residues putatively involved in saccharide binding are shaded. Conserved disulfide bridges are indicated at the bottom. In the consensus lines, "*" indicates identical or conserved residues in all sequences in the alignment; ":" indicates conserved substitutions; "." indicates semi-conserved substitutions. The 2D structure assignments were derived from the NMR structure of hevein (PDB: 1hev); the letters, "e", "h", and "t" represent "β-sheet", "α-helix", and "turn" or "coil" structure, respectively. The selected sequences were extracted from the following identified proteins or genes: Hevein (GI: 1070656), from *Hevea brasiliensis*; Avesin A, from *Avena sativa* (this study); CHIA MAIZE (GI: 283037), from *Zea mays*; CHIB SORGHUM (GI: 15529117), from *Sorghum bicolor*; ENDOCHI ARATH (GI: 15224321) and PR4 ARATH (GI: 15229342), from *Arabidopsis thaliana*; Ac-AMP2 (GI: 1703289), from *Amaranthus caudatus*; WIN2 POTATO (GI: 139699), from *Solanum tuberosum*; CBP20 (GI: 632736) and CHI2 TOBAC (GI: 116321), from *Nicotiana tabacum*; Pn-AMP1 (GI: 5902771), from *Pharbitis nil* (syn. *Ipomoea nil*); CHI2 ORYSA (GI: 116317), from *Oryza sativa*; CHI1 HORVU (GI: 2506281), from *Hordeum vulgare*; CHIA RYE (GI: 11344587), from *Secale cereale*; WGA (GI: 113505) A, B, C and D, from *Triticum aestivum*; UDA (GI: 4138900) A and B, from *Urtica dioica*.

growth medium, are essentially abolished in high-salt media. The antagonistic effect of salts is primarily due to the cations, in that divalent cations are more potent antagonists than monovalent cations (Broekaert et al., 1996; Huang et al., 2001; Koo et al., 1998). These features are similar to the initial phase (pore/ion-channel forming) of fungicidal effects of basic thionins and defensins (Broekaert et al., 1997; Florack and Stiekema, 1994). Impressively, hevein itself, with four positively charged and five negatively charged residues, has a pI value of 4.7, and is anionic, a feature similar to that of avesin A. In high concentrations (500–2000 μg/ml), hevein decreases the length of hyphae of the fungus *Pyrenophore tritici-repentis*, or totally inhibits its germination (Van Parijs et al., 1991). Although the antifungal activities of avesins in our experiments were not highly effective, we believe that avesins may comprise parts of the orchestrated cascades of plant defense, as does hevein (Broekaert et al., 1990; Raikhel et al., 1993).

We anticipate that avesins are post-translational cleavage products which may have tripartite precursors similar to those of hevein and Ac-AMPs that contain an N-terminal signal sequence, a domain corresponding to mature proteins, and a C-terminal extension (Broekaert et al., 1990, 1996; Raikhel et al., 1993). Oat is economically one of the most important of cereal foods, which worldwide contributes more than 70% of the calories in the human diet. Knowledge about defense genes in oat seeds could provide genetic insights into the construction of commercially more profitable hybrid seeds with improved yields (Crawley, 1999; Ferber, 1999; Lamtham and Day, 2000; Skadsen et al., 2000).

In addition, the identification of avesins (as hevein homologues) from oat seeds raises the question of whether avesins represent parts of the dust allergens which affect occupational workers and susceptible young children; although these events are rare (Block et al., 1984; Manfreda et al., 1986; Pazzaglia et al., 2000; Rasanen et al., 1994). Hevein has been recently identified as a major IgE-binding polypeptide in natural rubber latex, and has been "accused" of being the most common allergen, causing the latex-gloves related Ig-mediated hypersensitive reaction of health-care workers in hospitals (Chen et al., 1997, 1998). Oat is otherwise generally considered to be the safest foodstuff among cereals (wheat, triticale, rye, barley, and oat). Oat-originated in vitro antigenic prolamins (avenins, a group of grain proteins with high Glu/Pro content) have been reported (Egorov et al., 1994; Rocher et al., 1992), but their total amount in oat is lower than that found in other cereal species. Characterizing avesins from oat seeds requires further studies on their possible antigenic activity to genetically susceptible individuals. To investigate whether avesin A

homologues are present in other grain seeds is of further interest.

4. Experimental

4.1. Materials

The company Svenska Lantmännen, Sweden, provided the oat seeds (*Avena sativa* L.) cv Sang. Samples from that seed are kept at the Division of Pharmacognosy, Uppsala University (VM186). Acetonitrile (CH_3CN), *iso*-propanol (*i*-PrOH), and TFA were of HPLC grade. Purified water was generated by the MilliQ water purification system (Millipore, Bedford, MA, USA). The other reagents were of the highest analytical grade commercially available.

4.2. Extraction and isolation

Dried and powdered oat seeds (4.0 g) were pre-extracted four times with 40 ml dichloromethane (CH_2Cl_2) to remove lipophilic constituents. The air-dried residues were then extracted four times with 40 ml of solvent I (25% CH_3CN, 0.1% TFA in water). The extract was filtered through a 4.0 g polyamide gel (Riedel-de Haen, Germany) that was packed in a small glass column and pre-equilibrated with solvent I. The filtrate was evaporated to remove the organic solvents and then lyophilized to obtain 152.7 mg of the crude polypeptide fraction. This crude polypeptide fraction was then dissolved in 10 ml of solvent I, and diluted with 1–2 ml water. The conductivity of this solution was measured, and found less than that of solvent I (7.4 mS). The sample was subjected to solid-phase extraction using a PRS cation exchange column (5 g, Isolute PRS column, International Sorbent Technology Ltd, Mid Glamorgan, UK), which was washed with 50 ml of solvent I, followed by elution with 50 ml of solvent II (25% CH_3CN, 0.1% TFA, 0.5 M NaCl in water). The latter eluent was collected, concentrated, and then subjected to an Isolute C_{18} column (10 g, International Sorbent Technology Ltd, Mid Glamorgan, UK), eluted successively with 100 ml of 0.1% TFA in water, 50 ml of solvent I, 50 ml of solvent III (50% CH_3CN, 0.1% TFA in water), and 100 ml of solvent IV (80% CH_3CN, 0.1% TFA in water). The fractions from solvents I, III, and IV were pooled together and evaporated. Finally, 21.6 mg of polypeptide fraction, denoted as P fraction, was obtained after lyophilizing the collected fractions (yield 0.54%).

An initial analysis of the P fraction by RP-HPLC revealed a complex profile. That fraction was therefore subjected to high-performance cation exchange chromatography (ÄKTA basic system, Amersham Pharmacia Biotech, Uppsala, Sweden) using two coupled 50/7.5 (i.d.) mm Vydac Protein SCX columns (Hesperia, CA, USA). The column was eluted at a flow rate of 1.0 ml/min, first with 100% eluent A (15% CH_3CN, 0.1% TFA in water) for 10 min, and then with a linear gradient to 70% eluent B (15% CH_3CN, 0.1% TFA, 0.1 M NaCl in water) for 25 min, followed by 5 min isocratic washing with 70% eluent B. The UV-absorbance was simultaneously recorded at 215, 254, and 280 nm. Three fractions were collected manually according to the following t_R: fraction 1 (10–20 min); fraction 2 (25–30 min), and fraction 3 (33–38 min). Fraction 2, containing the highest amount of polypeptides (indicated by quantitative AAA), was further purified by repeated RP-HPLC using the Shimadzu LC10 system, equipped with a Shimadzu SPD-M10Avp DAD. In the final purification, a 250/4.6 (i.d.) mm Rainin Dynamax column (C_{18}, 5 μm, 300 Å) was used and eluted with a linear gradient of 5–40% organic modifier (CH_3CN/*i*-PrOH, 6/4) in 0.1% aqueous TFA for 50 min at a flow rate of 0.8 ml/min. The major polypeptide ($t_R = 35.7$ min) from fraction 2 was thus obtained.

4.3. Sequence determination and alignment

Quantitative AAA was performed at the Amino Acid Analysis Center, Department of Biochemistry, Uppsala University. In brief, the peptides were hydrolyzed for 24 h at 110 °C with 6 N HCl containing 2 mg/ml phenol, and the hydrolysate was analyzed with an LKB model 4151 Alpha Plus amino-acid analyzer using ninhydrin detection. Mass spectra were obtained using a Kratos Kompact IV MALDI-TOF mass spectrometer, or a Micromass Q-TOF electrospray instrument with nanospray source (Manchester, UK) in the positive mode. For the sequence analysis, the peptide was reduced with DTE in 0.25 M Tris–HCl containing 1 mM EDTA and 6 M guanidine–HCl (pH 8.5; 24 °C; 2 h). The reduced peptide was subsequently alkylated to its PEC derivative by adding 4-vinylpyridine to the solution (37 °C, 1 h). Reduction and alkylation were performed in the dark, under argon. The alkylated peptide was desalted and isolated using high-performance gel filtration on a Superdex Peptide HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden) eluted with 40% CH_3CN in 0.1% aqueous TFA. The amino acid sequence was then determined by automated Edman degradation using a protein sequencer (model ABI 476A; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

The determined amino acid sequence was used as an initial query for the PSI-BLAST search of the NR database at the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/blast/) with an expectation (*E*) value threshold = 0.001 (Altschul and Koonin, 1998; Altschul et al., 1997). The resulting statistically significant sequences were intentionally selected and

aligned with the CLUSTAL W program (http://www2.ebi.ac.uk/clustalw/) (Thompson et al., 1994). Sequence analyses were also conducted in the databases Pfam (http://www.sanger.ac.uk/Software/Pfam/) (Bateman et al., 2000; Sonnhammer et al., 1998) and SMART (http://SMART.embl-heidelberg.de/) (Ponting et al., 1999; Schultz et al., 1998, 2000).

Acknowledgements

We thank Drs. J.J. Borowicz and C.J. Welch from Agrivir AB, Uppsala, Sweden, for antifungal tests. We are very grateful to the Swedish Farmers Cooperative and VL-Foundation for financial support.

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